IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Soff *et al.*

Application No.: 08/991,761

Group Art Unit: 1642

Filed: December 16, 1997

Examiner: Minh-Tam B. Davis

For: METHODS AND COMPOSITIONS FOR GENERATING ANGIOSTATIN

Supervisory Examiner: Anthony C. Caputa
Attorney Docket No.: 10561-004

SUPPLEMENTAL DECLARATION OF GERALD A. SOFF, M.D., UNDER 37 CFR § 1.132

Honorable Commissioner of
Patents and Trademarks
Washington, D.C. 20231

Sir:

GERALD A. SOFF M.D. declares and states that:

1. I am a citizen of the United States, residing at 303 Apple Tree Lane, Wilmette, IL 60091.

2. I presently hold the position of Associate Professor of Medicine at Northwestern University Medical School, Chicago, IL, which position I have held since 2000. From 1991 to 2000, I held the position of Assistant Professor of Medicine, Northwestern University Medical School, Chicago, IL. I have held the positions of: Instructor in Medicine, Beth Israel Hospital, Harvard Medical School, Boston, MA; Visiting Scientist, Massachusetts Institute of Technology, Cambridge, MA; and Fullbright Scholar, Council of International Exchange of Scholars.

3. I received a B.A. from Johns Hopkins University in 1977 and an M.D. from Johns Hopkins School of Medicine in 1981. I completed a three-year internship/residency in medicine at the Medical College of Virginia and a three-year fellowship in hematology/oncology at Beth Israel Hospital, Harvard Medical School, Boston, MA. I am a Diplomat of The American Board of Internal Medicine, Subspecialty in Hematology.

4. My academic and technical experience, honors, and a list of my publications are set forth in my *curriculum vitae*, which was previously submitted on February 14, 2001 in my Declaration Under 37 C.F.R. § 1.132 as Exhibit A.

5. I am a coinventor of present U.S. Patent Application No. 08/991,761, filed 12/16/97, in the name of Soff *et al.* and entitled "Methods and Composition For Generating Angiostatin." I am familiar with the Office Action dated June 13, 2001 issued therein.

6. In the Office Action dated June 13, 2001, the Examiner rejected claims 59-80, based on a lack of enablement. During the Interview of October 10, 2001, the Examiner requested data showing the dose-response correlation between increasing levels of angiostatin and decreasing levels of cell or tumor growth. In addition, the Examiner also requested a Declaration showing that the administration of captopril alone (in the absence of plasminogen activator) would not result in antiangiogenic activity or in the generation of Angiostatin 4.5 in contrast to the teachings of Volpert *et al.* which suggests that captopril by itself induces antiangiogenic activity in rat corneas.

7. The following experiments have been performed. The results demonstrate: (1) the existence of a dose-response relationship of cell growth reduction due to increasing levels of angiostatin where the angiostatin was generated by treatment of human plasma with sulfhydryl donor and plasminogen activator; (2) that administration of captopril in the absence of plasminogen activator does not reduce the growth of tumors or induce the production of any detectable levels of Angiostatin 4.5 or other angiostatin isoforms in mice *in vivo*; (3) that administration of captopril in the absence of plasminogen activator to human plasma *ex vivo* does not generate any detectable levels of Angiostatin 4.5; and (4) that administration of a plasminogen activator alone to human plasma *ex vivo* did generate a small amount of Angiostatin 4.5.

Dose-Response Relationship of Angiostatin And Cell Growth Reduction

8. An endothelial cell proliferation assay was performed. *See* Figure 1, Exhibit 1. Bovine aortic endothelial cells (<passage 15) were plated in 24-well culture dishes at 10,000 cells/well in DMEM supplemented with 2.5% heat-inactivated calf serum, 1% penicillin-streptomycin, in a standard humidified incubator. The following day, fresh medium supplemented with 3 ng/ml human basic fibroblast growth factor (bFGF) (Becton Dickinson,

Bedford, MA) alone, or bFGF supplemented with pure human Angiostatin4.5. Various concentrations of human Angiostatin4.5 were tested. At 24 and 48 hours the media was replenished. After 72 hours treatment, the cell number was determined by counting from duplicate wells.

9. Exhibit 1 illustrates the results from the cell proliferation assay showing that as the concentration of angiostatin levels increase, cell growth in the assay decreases (the angiostatin being generated by treatment of human plasma with sulfhydryl donor and plasminogen activator). Inhibition begins to occur with as little as 10 to 25 nM of angiostatin, significantly increasing thereafter as the concentration of angiostatin reaches 100 nM, demonstrating a biologically relevant range.

Treatment of Mice Tumors With Sulfhydryl Donors

10. Female beige nude mice (approximately 25gm each) were treated with a EOMA cells from a human hemangioendothelioma. Each mouse was injected subcutaneously with 0.5 x 10^6 EOMA cells on 10/25/1999. There were four mice per treatment and control cohort. Tumor size was measured at regular intervals. At completion of the experiment, mice were sacrificed and plasma prepared, as described below.

11. Treatment began on 10/26/1999, 24 hours after the cells were injected. Test mice received 1.25 mg of captopril diluted in 0.5 ml saline, subcutaneously, twice daily. The 1.25 mg dose of captopril was chosen based on Volpert *et al.*, 1996 in which 50 mg/kg per day was used in tumor-bearing rats (which is equivalent to 1.25 mg per mouse, averaging 25 gm). This dose, administered subcutaneously twice daily, was thought to provide more predictable dosing than oral dosing in the drinking water as in Volpert. Thus the dose was at least as great an exposure to captopril compared to Volpert *et al.* Control mice received 0.5 ml of saline that was injected subcutaneously, twice daily.

12. Mice were treated for the specified number of days and sacrificed per protocol. Whole blood was collected, anticoagulated with 10 mM EDTA, and platelet-poor plasma was prepared by centrifugation (5000g at 4°C, for 15 minutes) and frozen at -20°C until use.

13. A western blot analysis was performed on the mouse plasma. The plasma samples were diluted 1:500 in Tris-Glycine running buffer, and electrophoresed under non-reducing conditions on 12% polyacrylamide gels (NOVEX, San Diego, CA) in Tris-Glycine

running buffer. The polyacrylamide gels were electrotransferred to a polyvinylene difluoride (PVDF) membrane (Immobilon, Millipore, Bedford, MA). The membrane was then blocked for >30 minutes in blocking buffer (1% bovine serum albumin in Tris-buffered saline) and probed with a conjugate of GMA086 (Green Mountain Antibodies, Vermont) and alkaline phosphatase (Sigma). GMA086 has been shown in preliminary data to bind to the kringle domains of plasminogen and angiostatin. Blots were developed using 5-bromo-4-chloro-3-indoyl-phosphate/nitroblue tetrazolium (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Direct conjugation of the primary antibody avoids the artifact of a second antibody (i.e. rabbit antimouse IgG-alkaline phosphatase) which could cross-react with the human IgG in the plasma samples.

14. Tumor size was measured in all mice during the experiment. As shown in Figure 2, Exhibit 2, administration of captopril had no antitumor effect on tumor-bearing mice. These data indicate that the antiangiogenic effect of the Angiostatic Cocktail is not reproduced by administration of captopril alone. In contrast, subcutaneous administration of 1 mg of pure Angiostatin 4.5, administered twice daily to mice with EOMA tumors, has been shown to be associated with suppression of tumor growth. *See* Figure 3, Exhibit 3. These data have been previously published by Soff and colleagues (*see* Lannutti *et al.* 1997).

15. To detect possible conversion of mouse plasminogen to Angiostatin 4.5 *in vivo*, induced by captopril, plasma was prepared from mice at completion of the experiment, and assayed by western blot. As shown in Figure 4, Exhibit 4, no angiostatin was detected by administration of captopril alone.

16. Thus, administration of captopril to mice, by itself, was not associated with the generation of detectable levels of Angiostatin 4.5, nor other isoforms of angiostatin. Furthermore, the administration of captopril to mice, by itself, was not associated with a reduction in tumor growth rates. The data indicate that the generation of Angiostatin 4.5 by the Angiostatic Cocktail is not due to activity of the free sulfhydryl donor captopril alone, nor by some particular property of captopril itself. Thus, the above-identified mouse experiment demonstrates the unexpected result that captopril does not exhibit antiangiogenic activity in mice when administered alone in the absence of plasminogen activator or Angiostatin 4.5.

Ex Vivo Conversion of Plasminogen To Angiostatin By a Plasminogen Activator and a Free Sulfhydryl Donor

17. In addition, to the above-identified experiments, another experiment was conducted to determine the relative contributions of a plasminogen activator and a free sulfhydryl donor such as captopril to the conversion of plasminogen to Angiostatin 4.5 in human plasma.

18. 1 ml of human plasma (anticoagulated with 10 mM EDTA) was treated with a plasminogen activator (recombinant urokinase, provided by Abbott Laboratories) or a free sulfhydryl donor (captopril or N-acetyl cysteine), or both plasminogen activator and free sulfhydryl donor. As control, plasma was incubated with no added reagents. At several time points (0, 2, 5, and 18 hours), 100 ul aliquots were removed and assayed by western blot for the conversion of plasminogen to Angiostatin 4.5.

19. A western blot analysis of the plasma was performed. The plasma samples were diluted 1:120 in Tris-Glycine running buffer, and electrophoresed under non-reducing conditions on 12% polyacrylamide gels (NOVEX, San Diego, CA) in Tris-Glycine running buffer. The polyacrylamide gels are electrotransferred to a polyvinylene difluoride (PVDF) membrane (Immobilon, Millipore, Bedford, MA). The membrane was then blocked for >30 minutes in blocking buffer (1% bovine serum albumin in Tris-buffered saline) and probed with a conjugate of GMA086 (Green Mountain Antibodies, Vermont) and alkaline phosphatase (Sigma). GMA086 has been shown in preliminary data to bind to the kringle domains of plasminogen and angiostatin. Blots were developed using 5-bromo-4-chloro-3-indoyl-phosphate/nitroblue tetrazolium (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Direct conjugation of the primary antibody avoids the artifact of a second antibody (i.e. rabbit antimouse IgG-alkaline phosphatase) which could cross-react with the human IgG in the plasma samples.

20. There were 6 sets of plasma incubates: (1) Plasma 1ml only; (2) Plasma + uPA 200 IU/ml; (3) Plasma + NAC 50 uM; (4) Plasma + uPA 200 IU/ml + NAC 50 uM; (5) Plasma + Captopril 50 uM; and (6) Plasma + uPA 200 IU/ml + Captopril 50 uM.

21. The results in Figure 5, Exhibit 5, indicate that incubation of plasma alone had no effect. Incubation of plasma with uPA did result in the formation of plasmin from plasminogen and complexes of plasmin with its inhibitors α–2-Antiplasmin and α-2-macroglobulin. In addition, incubation of plasma with uPA resulted in the detection of trace levels of Angiostatin 4.5.

22. When plasma was incubated with captopril alone, no Angiostatin 4.5 was generated. These experiments included evaluation of 50 uM captopril (Figure 6, Exhibit 6). If 200 IU/ml of recombinant human urokinase (uPA) was added to the plasma and captopril, than Angiostatin 4.5 was generated with time along with a reduction in plasminogen/plasmin levels.

23. These western blot studies indicate that for the conversion of plasminogen to Angiostatin 4.5 in human plasma *ex vivo*, captopril alone is insufficient. A plasminogen activator, such as uPA is also required. This study specifically demonstrates the unexpected result that captopril alone is insufficient to generate antiangiogenic activity including Angiostatin 4.5 or other angiostatin isoforms which have antiangiogenic activity.

24. I declare further that all statements made in this Declaration of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Respectfully submitted,

Date 12/4/2001

Gerald A. Soff, M.D.

Attachments:

Exhibit 1: Figure 1
Exhibit 2: Figure 2
Exhibit 3: Figure 3
Exhibit 4: Figure 4
Exhibit 5: Figure 5
Exhibit 6: Figure 6

EXHIBIT 1

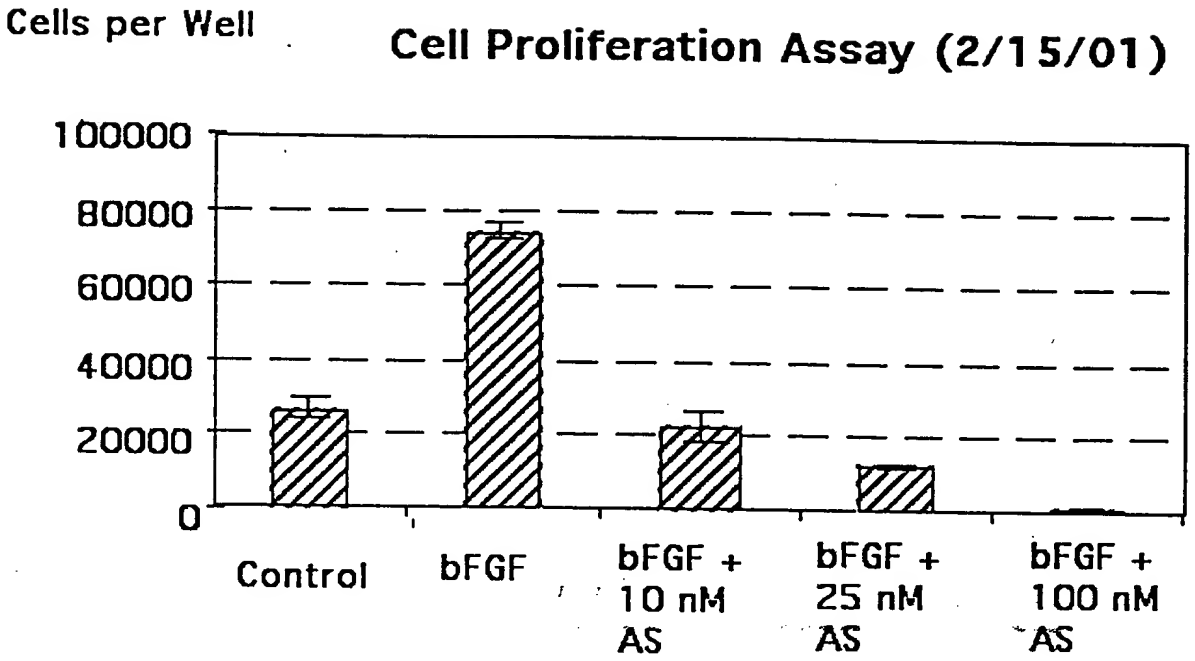
Cells per Well
Cell Proliferation Assay (2/15/01)
100000
80000
60000
40000
20000
0
Control
bFGF
bFGF + 10 nM AS
bFGF + 25 nM AS
bFGF + 100 nM AS

Figure 1

EXHIBIT 2

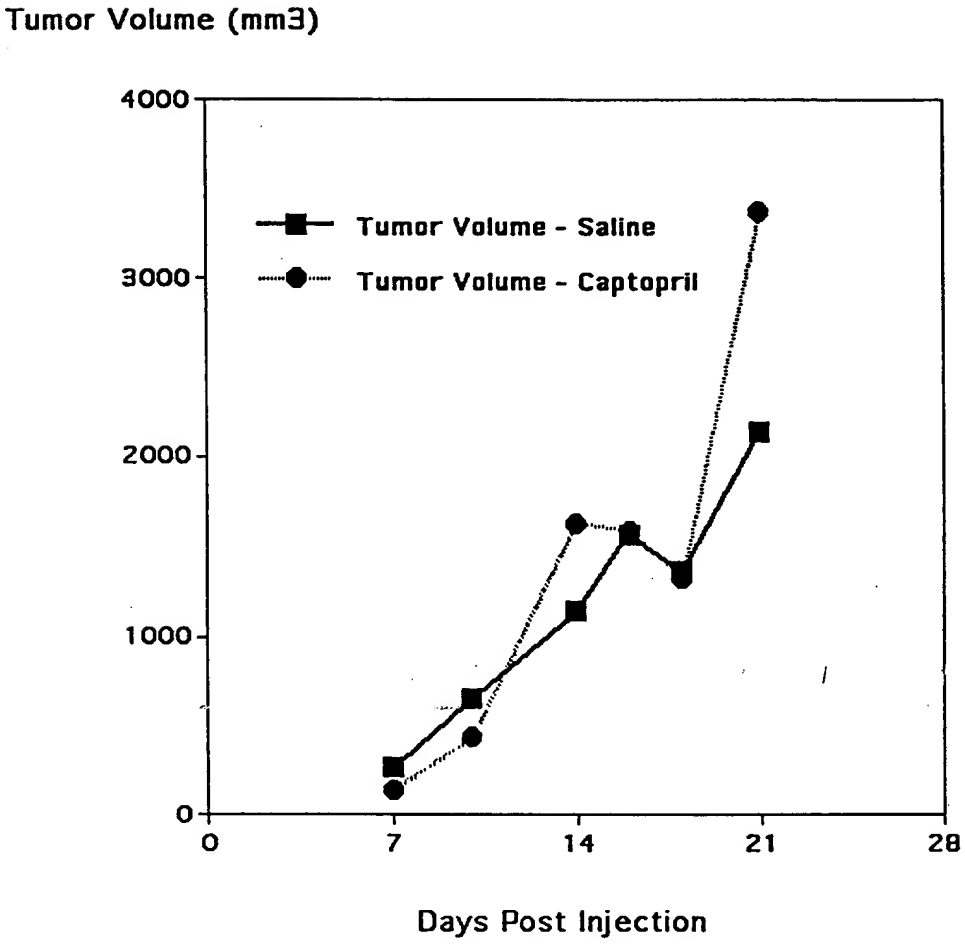
Tumor Volume (mm3)
4000
3000
2000
1000
0
Tumor Volume - Saline
Tumor Volume - Captopril
0
7
14
21
28
Days Post Injection

Figure 2

EXHIBIT 3

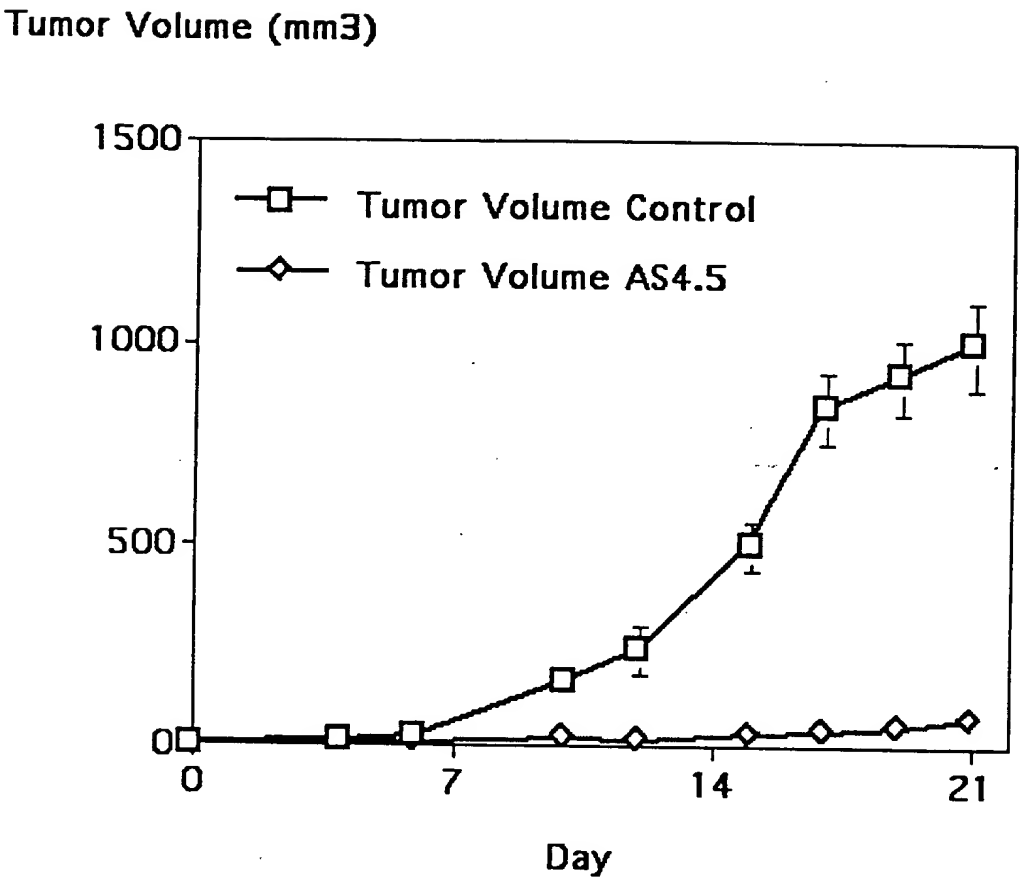
Tumor Volume (mm3)
1500
1000
500
0
Tumor Volume Control
Tumor Volume AS4.5
0
7
14
21
Day

Figure 3

EXHIBIT 4

Figure 4. Western Blot Of Mouse Plasma

Using a monoclonal antibody to the kringle domain of plasminogen, plasminogen and Angiostatin4.5 is detected. In mice treated with captopril (lanes 1-3) no Angiostatin4.5 was detected, as with saline control mice (lanes 4 ,5). Lane 6 was 20 ng of pure human AS4.5, as a control to compare to the *in vivo* detected angiostatin.

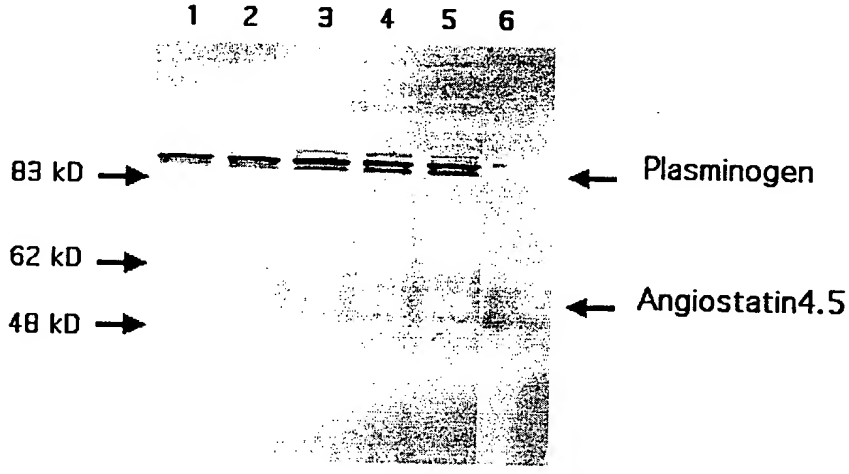

EXHIBIT 5

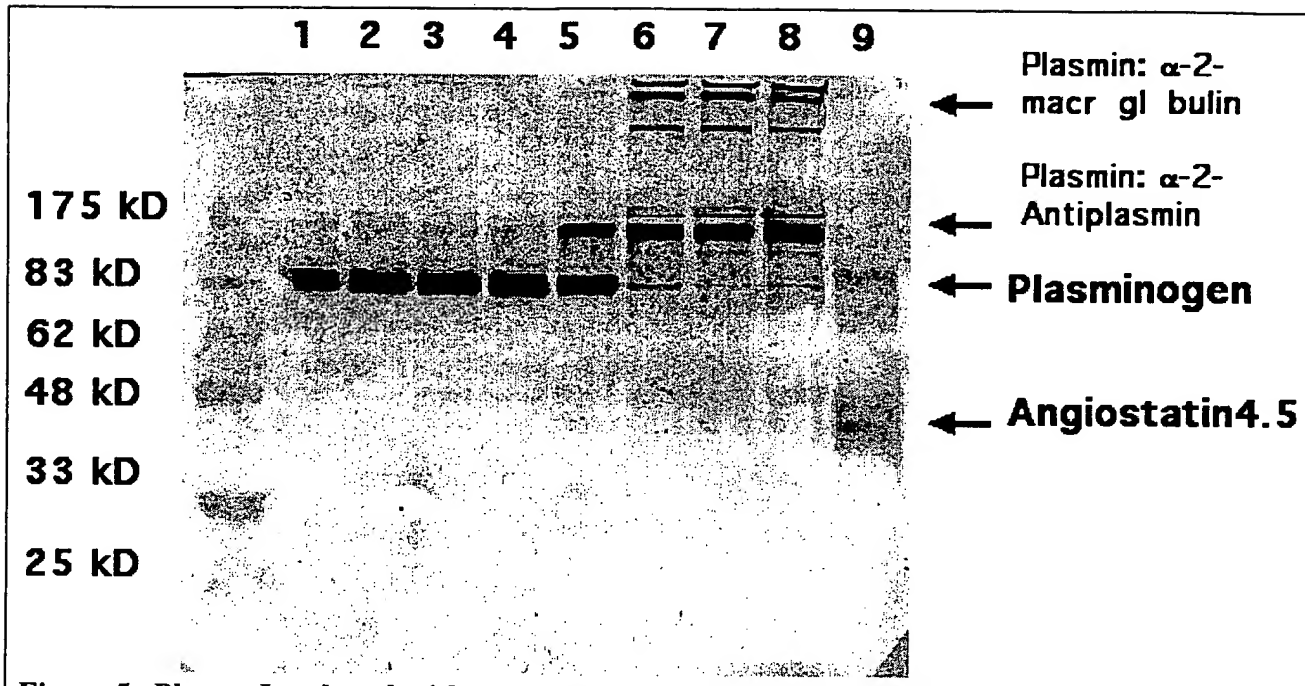

Figure 5. Plasma Incubated with no Additives or uPA alone (200 IU/ml) Incubation of plasma alone had no effect. Incubation of plasma with uPA resulted in formation of plasmin from plasminogen and complexes of plasmin with its Serpin inhibitors, α-2-antiplasmin and α-2-macroglobulin. Trace levels of Angiostatin4.5 were detected in this reaction, indicating that a plasminogen activator alone may generate Angiostatin4.5 in plasma, but not as efficiently as with addition of a free sulfhydryl donor.

Lane 1; Control plasma; 0 hr.
Lane 2; Control plasma; 2 hr
Lane 3; Control plasma; 5 hr
Lane 4; Control plasma; 18 hr
Lane 5; Plasma + uPA; 0 hr.
Lane 6; Plasma + uPA; 2 hr.
Lane 7; Plasma + uPA; 5 hr.
Lane 8; Plasma + uPA; 18 hr.
Lane 9; Pure Angiostatin4.5 (100 ng).

EXHIBIT 6

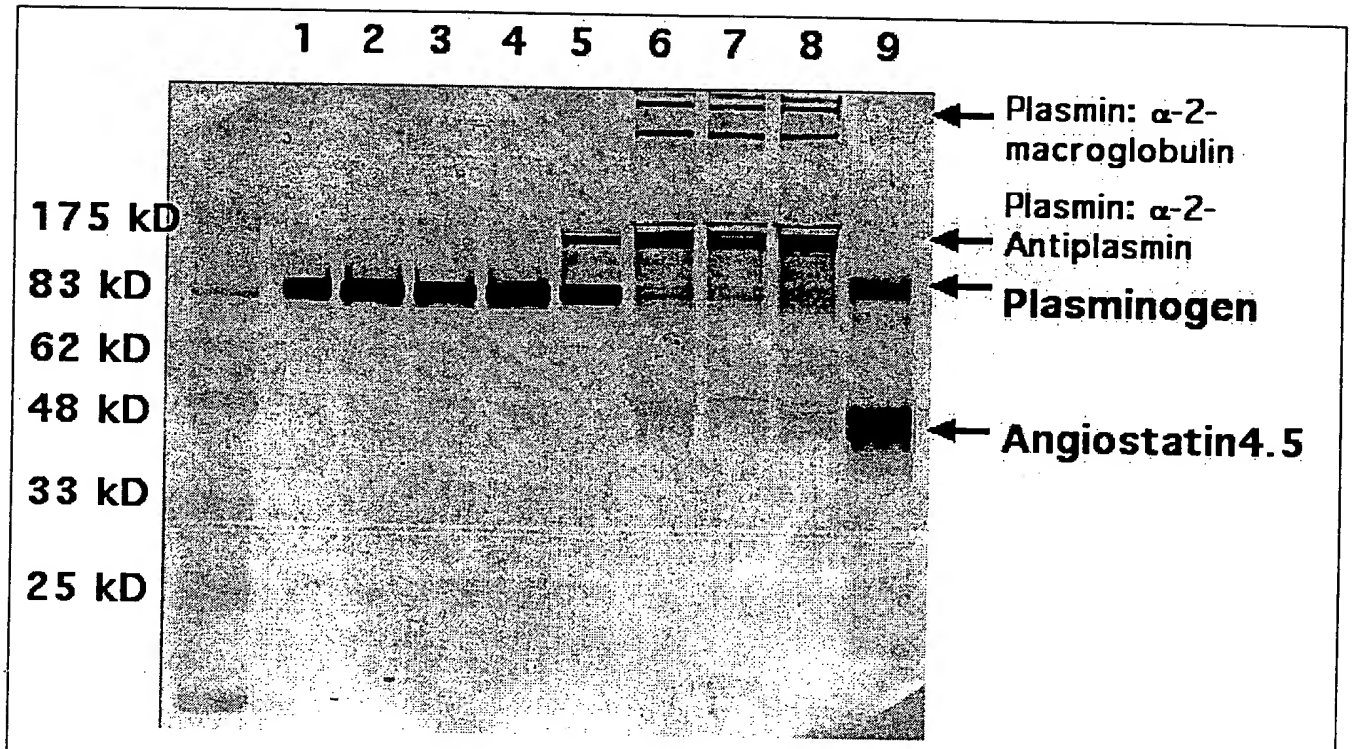

Figure 6. Plasma Incubated with Additives of Captopril or captopril and uPA. Incubation of plasma with Captopril alone did not result in detectable levels of Angiostatin4.5 or other angiostatin isoforms. However, incubation of captopril and uPA together did result in conversion of plasminogen to Angiostatin4.5 in plasma (lanes 5 – 8).

Lane 1; Plasma + Captopril; (50 uM); 0 hr.
Lane 2; Plasma + Captopril; (50 uM); 2 hr
Lane 3; Plasma + Captopril; (50 uM); 5 hr
Lane 4; Plasma + Captopril; (50 uM); 18 hr
Lane 5; Plasma + Captopril; (50 uM) + uPA (200 IU/ml); 0 hr.
Lane 6; Plasma + Captopril; (50 uM) + uPA (200 IU/ml); 2 hr.
Lane 7; Plasma + Captopril; (50 uM) + uPA (200 IU/ml); 5 hr.
Lane 8; Plasma + Captopril; (50 uM) + uPA (200 IU/ml); 18 hr..
Lane 9; Pure Angiostatin4.5 (100 ng).